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TITLE: Exploring AR-NFkappaB/p52-Targeted Inhibitors as Novel Therapy Against Castration-Resistant Prostate Cancer Progression

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14. ABSTRACT The goal of this research was to verify the specificity of the inhibition of androgen receptor (AR) – NFkB/p52 interaction by small molecule AR/p52 inhibitors (selected from prior high throughput screen) in cell culture and AR/p52 activity assays, and determine the efficacy of the compounds against castration resistant prostate cancer (CRPCa) cell / xenograft growth. Data from this research should establish the AR-p52 interaction as a viable new target for preventing progression to CRPCa and identify lead compound(s) to be further developed for preclinical toxicity testing and clinical trials for PCa that fall beyond the scope of this proposal. Cell culture studies identified a lead compound with IC_{50} of 5 μM against both androgen-dependent and –independent PCa cell growth that involves reduction in CyclinD1, and significant inhibition of AR transcriptional activity as measured by PSA mRNA as well as inhibition of AR and p52 translocation to nucleus. Efficacy of the lead compound in an oral regimen against CRPCa xenograft tumor development and progression was established. Thus the lead compound shows evidence of specificity for AR-p52 interaction and efficacy against CRPCa. These data help establish AR-p52 interaction as a viable new target for PCa therapy and identify a lead compound that can be further developed toward clinical application as a new therapeutic agent to prevent progression of CRPCa.

15. SUBJECT TERMS androgen receptor, nuclear factor kappa B p52, small molecule inhibitors, human prostate carcinoma cells, castration resistant prostate cancer xenograft efficacy

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INTRODUCTION

About 30% of all prostate cancer (PCa) patients after first line of therapy succumb to recurrent PCa. Although the recurrent PCa regresses after androgen deprivation therapy (ADT), the majority of these patients return to the clinic with the refractory PCa known as castrate resistant PCa (CRPCa). There is no approved drug that can prevent the transition of PCa to CRPCa in these patients. Accumulative evidence suggests that androgen-independent activation of androgen receptor (AR) and development of apoptosis-resistant cells play key roles in the transition of androgen-dependent PCa (ADPCa) to CRPCa (1). AR activation and signaling under very low androgen level or in the absence of androgen may occur by a variety of mechanisms that alter the sensitivity and/or specificity of AR activation. Most of the NF-κB proteins have been shown to be aberrantly activated in PCa cells and tissues (2). In the less-explored non-canonical NF-κB2 pathway, protein p52 induces the expression of genes that are involved in hyperplasia, growth and cell proliferation. Overproduction of p52 has been observed in several solid tumors including PCa, and it was recently shown that overexpression of p52 induces castration-resistant growth in human prostate carcinoma LNCaP cell xenografts by inhibiting both cell cycle arrest and apoptotic cell death induced by androgen deprivation (3). Thus, one possible mechanism of aberrant activation of AR in the absence of androgen is its activation by p52. Using a *Gaussia* Luciferase (GL) reconstitution assay (4), we found that AR interacts directly with p52 *in situ* under androgen-deprived conditions. We further developed and used a novel GL reconstitution based high throughput screening assay to identify four drug-like small molecules that specifically inhibit this interaction of AR and p52. We successfully used this method for identifying the inhibitors of AR-JunD interaction (5). First, we performed a preliminary high throughput screen (HTS) of a 2,800 compound subset of the Life Chemicals Library of drug-like small molecules (6). This screen was performed under an androgen-deprived condition and yielded 296 “hits” which were further screened using a positive control reported in the literature (4) to eliminate the non-specific inhibitors. Only “hits” that inhibited GL-reconstitution in cell lysate from GL1-AR/P52-GL2 co-transfection, but failed to inhibit the control protein-protein interaction were considered “true” hits. These prior studies yielded four compounds that are specific inhibitors of AR and p52 in the absence of androgen (shown in Figure 1 in Appendix). We hypothesize that small molecule inhibitors of the AR-p52 interaction will inhibit aberrant activation of AR by p52 and thereby prevent the transition of ADPCa to CRPCa and growth of CRPCa. The purpose of this research is to verify the specificity of the inhibition of AR-p52 interaction by these compounds in cell culture as well as in AR/p52 activity assays, and investigate the anti-CRPCa activity of the compounds in androgen-independent human LNCaP C4-2 cells and xenografts. Data from this research will help identify lead small molecule(s) to be further developed for preclinical toxicity testing and clinical trials for PCa that fall beyond the scope of this proposal. This report summarizes the progress that has been achieved toward completing the proposed aims.

BODY

The following are the data collected with respect to tasks listed in our statement of work (all figures and tables referred to in this report are included in the Appendix:

Task 1. Investigate the anti-CRPCa activity and specificity of the inhibition of AR-p52 interaction by the four HTS-identified drug-like small molecule AR-p52 inhibitors *in vitro*:

Effect of the AR-p52 inhibitor compounds on the growth of androgen-dependent LNCaP and the castration resistant variant LNCaP C4-2 cells:

We have determined the effects of these four inhibitors on the growth of androgen-dependent LNCaP cells and its castration resistant variant LNCaP-C4-2 cells. Analysis of the effect of these compounds on the castration-resistant (CR) growth of LNCaP C4-2 cells under androgen

deprivation conditions using our published assay (7) showed significant (P<0.05) growth inhibition by all four inhibitors in the 1 to 25μM dose range, and compounds AR/p52-01, -02 and -03 had IC_{50}s of 10μM or less (Table 1, see Appendix). Danquah *et al* (8) showed that the IC_{50} of the clinically used bicalutamide (Casodex, Astra-Zenecca; an androgen antagonist that is the current standard ADT) in LNCaP C4-2 cells is 92.9 μM, which is likely a clinically unachievable concentration. Thus, these small molecule inhibitors of AR-p52 interaction show promise for blocking CRPCa growth post ADT. Similar analysis of effect on androgen-dependent (AD) growth of the parental LNCaP cells under growth stimulatory androgen conditions showed AR/p52-01 and -02 had IC_{50}s of 5μM or less (Table 2, see Appendix), with significant growth inhibition in the 1 to 25μM dose range, while compounds ARp52-03 and -04 showed no significant effect on AD LNCaP growth at any dose. Overall, compounds AR/p52-01 and -02 were the most effective for inhibiting these models of PCa cell growth, with IC_{50}s at 4 to 5 μM for both CR LNCaP C4-2 and AD LNCaP growth. Lead compound AR/p52-02 was also tested against another CRPCa cell model, 22Rv1 PCa cells, and found to have an IC_{50} of 10 μM in these cells (noted in Table 2), thus verifying inhibitory activity of lead compound AR/p52-02 against CRPCa cell growth.

The AR-p52 inhibitors do not bind to the Ligand Binding Domain (LBD) of AR:
It has been shown that NF-κB2/p52 and N-terminal domain (NTD) of AR co-immunoprecipitate (9). Based on this finding, we hypothesized that the inhibitors of this interaction may interfere with binding of NF-κB/p52 to NTD portion of AR and not to its Ligand Binding domain (LBD). We performed a Ligand Binding Competition Assay (using Polarscreen™ AR Competitor Assay kit from Invitrogen). In Figure 2 (see Appendix), we show that these inhibitors do NOT compete with androgen for binding to the LBD of AR and therefore we concluded that the inhibition of interaction between AR and p52 by these inhibitors is not due to their interference with the binding to LBD of AR. Lack of interference with androgen for binding to AR supports the specificity of these compounds for AR-p52 interaction.

The AR-P52 inhibitors reduce the expression of PSA in androgen-dependent LNCaP cells as well as castration resistant LNCaP C4-2 cells:
As PSA protein expression is a marker of AR activity, we performed first a preliminary analysis to determine the ability of these inhibitors to inhibit PSA expression in castration resistant LNCaP-C4-2 cells (data are summarized in Table 3, see Appendix). Compound AR/p52-02 showed the greatest effect in inhibiting PSA secretion with PSA secretion significantly decreased to 22% of control (P<0.02). Interestingly, LNCaP C4-2 cells had 15-fold greater PSA secretion compared to parental LNCaP cells under the same androgen-deprived conditions, with values of 6.2 ng PSA/cell and 0.4 ng PSA/cell respectively in this study. These data indicated significant AR transcriptional activity in androgen-independent LNCaP C4-2 cells under androgen-deprived conditions and the ability of the AR-p52 inhibitors to block this activity.

As shown in Figure 3 (see Appendix), LNCaP (top graphs) and C4-2 (bottom graphs) cells were grown overnight in androgen-deprived medium, then treated with 5μM (IC50) or 10 μM of AR/p52-02 (F1174-3266) (D) in the presence or absence (±) of 2nM synthetic androgen R1881 (R). After 72h, the cells were collected using Invitrogen's Cells-to-cDNA kit for quantitative real time PCR analysis using CFX96 instrument (BioRad) to evaluate the level of expression of PSA from each condition. The sequence of PSA primers used are as follows:

PSA forward:5'GACCACCTGCTACGCCTCA PSA reverse:5' GGAGGTCCACACTGAAGTTTC. The CT value of PSA was normalized against 18S rRNA cDNA. The sequence of primers used

for 18S rRNA in real time PCR were as follows; 18S rRNA forward: 5'CGCCGCTAGAGGTGAAATCT and reverse sequence:5'CGAACCTCCGACTTTCGTT. AR/p52-02 significantly reduces AR transcriptional activity as measured by PSA expression in LNCaP cells under low androgen (F1C4) condition but does not affect androgen induction of AR transcriptional activity: AR/p52-02 significantly reduced PSA mRNA under the androgen-deprived condition ($P<0.05$ for -R,-D compared to –R,+D at all timepoints) in both LNCaP and C4-2 cells, with greater effect at 10 μM compared to 5μM. As expected, 2nM androgen stimulation led to a significant ~16-fold increase in PSA mRNA ($P<0.001$ for +R,-D compared to –R,-D at all timepoints) for LNCaP cells. C4-2 cells also responded to androgen stimulation, but more mildly with only ~3-fold increase ($P<0.01$ for all comparisons). However, AR/p52-02 did not affect PSA mRNA levels under the 2nM androgen-stimulated condition, as no difference was observed at any timepoint for +R,-D compared to +R,+D. Interestingly, baseline PSA mRNA was about 6-fold higher in C4-2 cells compared to LNCaP cells. (N=6 per condition between two experiments).

The level of PSA expression was not decreased in CWR22v1 cells as shown in Figure 4 (see Appendix). This might be due to the low activity of Androgen receptor in these cells as the mutations in AR rendered AR inactive and not responding to androgen treatment until later time point (72h).

We also looked at the level of expression of PSA in C4-2 cells after treatment with AR/p52-01 (F1174-2988) in the presence and absence of 2nM R1881 (Figure 5, see Appendix)) and did not see any reduction of PSA mRNA after treatment with this inhibitor, therefore we did not pursue studying the mechanism of action of this drug further.

Further analyses of the lead compound AR/p52-02, based on cell growth and PSA analyses, were performed to explore its mechanism of action *in vitro*.

Lead compound AR/p52-02 does not affect apoptosis marker Cleaved PARP, but does affect cell cycle marker Cyclin D1:

Western blot analysis for effect of lead inhibitor AR/p52-02 on a marker of apoptosis, Cleaved PARP, and a marker of cell cycle, Cyclin D1 was performed in LNCaP and LNCaP C4-2 cells (Figure 6, see Appendix). There was no effect on cleaved PARP, suggesting the mechanism of action of AR/p52-02 does not involve apoptosis. Western blot analysis of Caspase 3 and Caspase 7 also showed no effect, further supporting that apoptosis is not involved. However, the reduction of Cyclin D1 in C4-2 cells under androgen-deprived condition as well as in both C4-2 and LNCaP in the presence of androgen suggests the mechanism of action of AR/p52-02 involves cell cycle.

Importantly, western blot analysis of LNCaP cells treated with or without AR/p52-02 at IC_{50} value of 5 μM showed no effect of the drug on the total AR or p52 protein levels, however it affects the translocation of each protein as shown in (Figure 7, see Appendix). The translocation of both p52 and AR is substantially inhibited in 5μM and 10μM of the inhibitor indicating that although the total level of AR and p52 remains the same across the different treatments when compared to the control, the interaction and activation (nuclear translocation) of both proteins are affected by the treatment with AR/p52-02 that supports the specificity of the compound for the inhibition of interaction of AR with p52.

Based on the results of Task 1, compound AR/p52-02 was prioritized as the lead compound for

the proposed *in vivo* studies in Task 2.

Task 2. Investigate the anti-CRPCa activity of up to two compounds selected from Task 1 *in vivo* .

Determination of oral bioavailability and maximum tolerated dose (MTD) for lead compound AR/p52-02:
Compound AR/p52-02 was advanced into animal studies. Solubility of the compound was determined in our standard administration vehicle, 0.9% saline. A preliminary PK / toxicity study indicated that a single dose of 5 mg/kg in 6%DMSO saline by either intra venous (IV) or oral administration was well-tolerated and yielded plasma levels at 30 minutes post administration of 3.2nM Cmax for IV and .05nM Cmax for oral dosing (Table 5A, see Appendix). Thus, the compound was determined to have oral bioavailability, and is therefore an ideal candidate for clinical application. Additional PK and maximum tolerated dose (MTD) studies for a daily oral dosing regimen of this compound were undertaken. MTD studies established the regimen of 50 mg/kg oral dailyx5 every week, using a 60% DMSO in saline formulation, as MTD for use in efficacy studies. The second PK study (Table 5B, see Appendix) demonstrated that a single 50 mg/kg oral dosing yielded a plasma level of 2nM AR/p52-02 at 2 hours post administration, which was 10-fold greater than the 0.1nM plasma level for a single 5 mg/kg oral dosing in the same study. AR/p52-02 was completely cleared from the plasma for both dosing regimens by 24 hours post administration. Plasma samples from MTD study mice harvested after 4 weeks of the dailyx5 per week 50 mg/kg oral treatment at the nadir timepoint, i.e. two days post final treatment, also showed no detectable levels of AR/p52-02 in plasma (N=8). Overall our data show that we achieved nM level of AR/p52-02 in the plasma with the 50 mg/kg oral dosing, but the compound was cleared from the blood within 24 hours and did not accumulate over multiple treatments. We plan to determine tissue levels of drug in future analyses.

Efficacy of AR/p52-02 against castration-resistant LNCaP C4-2 xenografts compared to parental androgen-dependent LNCaP xenografts:
Final data from studies of the efficacy of AR/p52-02 (F1174-3266) against the castration resistant LNCaP C4-2 xenograft model of prostate cancer compared to the parental androgen dependent LNCaP model are shown in Figure 8 (see Appendix). The drug showed significant effect in reducing the development and progression of C4-2 tumors with no apparent toxicity, and therefore demonstrated efficacy against this animal model of CRPCa. In contrast, AR/p52-02 was not effective against the parental androgen-dependent LNCaP xenograft tumors, though significantly greater body weights and overall body condition of mice treated with drug suggested some benefit of drug in this model. Analyses of effect of the drug on markers in the C4-2 tumors could not be completed due to an insufficient number of tumors to analyze: the strong effect of the drug against tumor development yielded just two mice with tumors (out of 30 mice total) among the drug-treated C4-2 groups. However, the striking effect against C4-2 tumor development and progression, under both castrated and intact conditions, establishes the anti-CRPCa activity of AR/p52-02 *in vivo* . The lack of effect of the drug against LNCaP tumors may be due to the growth of those tumors being driven by androgen activation of AR, which may initiate a very robust / different growth signaling pathway that could not be competed against by this drug. However, the fact that this drug was able to inhibit the C4-2 cells even under the androgen intact condition further supports the utility of this drug for prevention of proliferation of a castrate-resistant cell population in men with PCa.

KEY RESEARCH ACCOMPLISHMENTS:

- Based on the studies on the level of PSA expression and growth inhibitory effects of all four compounds by determining their IC_{50}s, we identified **AR/p52-02 (F1174-3266)** as the **lead compound** for further studies on the mechanism of action of the drug.
- By western blot analysis of whole cell lysates of both LNCaP and its castrate resistant variant (C4-2), we determined that AR/p52-02 reduces the growth of both cell lines by reducing the expression of CyclinD1 and not by inducing the apoptosis pathway.
- We further determined that although the lead compound did not change the total level of either of proteins (AR or p52), it *did* inhibit the translocation of these two transcription factors to the nuclei that further supports the notion that inhibition of interaction of these proteins causes reduction in cross talk and transcriptional activity of both proteins.
- By *in vivo* studies we established that lead compound AR/p52-02 has oral bioavailability, and is well-tolerated and effective against development and progression of castrate-resistant C4-2 xenografts in mice at the MTD of 50 mg/kg administered orally once daily x 5 every week.
- Overall we established that AR-p52 interaction is a viable new target for PCa therapy and identified inhibitor AR/p52-02 as the lead compound to be further developed toward clinical trials as a new therapeutic agent for PCa.

REPORTABLE OUTCOMES:

- Siefkes E, Church D, Wilding G, and Mehraein-Ghomi F. The effect of four inhibitors of androgen receptor and p52 interaction on the proliferation of prostate cancer cells. Poster Presentation at UW-Madison Biology Undergraduate Mentored Research Symposium, May 2012.
- Nuandorf M, Church D, Mehraein-Ghomi F, Wilding G. Efficacy of a novel drug against human prostate cancer xenografts. Poster Presentation at UW-Madison Undergraduate Mentored Research Symposium, May 2014.
- Mehraein-Ghomi F, Church D, Naundorf M, Schreiber C, Siefkes E, Weichmann A, Basu H, Wilding G. Specific inhibitor of androgen receptor and NF-κB/p52 for prevention of castration-resistant prostate cancer progression (manuscript in preparation)

CONCLUSION:

In conclusion, the data overall demonstrate a specificity of these compounds for inhibiting the AR – p52 interaction and efficacy in blocking both androgen-dependent and androgen-independent (castration resistant) prostate cancer cell growth. The lead compound, AR/p52-02 (F1174-3266), showed efficacy against growth of castration resistant human prostate cancer xenografts in an oral regimen, and is therefore an excellent candidate for further preclinical development toward clinical testing. The data also establishes AR-p52 inhibitors as a new class of agents for further research and development as new therapies to prevent progression to CRPCa in PCa patients who underwent androgen ablation therapy. A provisional patent is in place for these compounds and we are in discussion with a drug company to pursue full patent and development of lead compound F1174-3266. We also intend to pursue additional funding to explore the mechanism of action of the lead compound, including additional studies to look at markers in animal models and studies to further delineate the AR-p52 signaling pathway in PCa development and progression, which will aid in development of compounds for clinical application as well as prognostic biomarkers to identify patients who will most benefit from AR-p52 targeted therapies.

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BIBLIOGRAPHY

None (manuscript in preparation noted above)

PERSONNEL

Full-time personnel who received pay from this research effort include:

Basu, Hirak S.

Church, Dawn R.

Mehraein, Farideh

Weichmann, Ashley M.

Wilding, George

APPENDIX

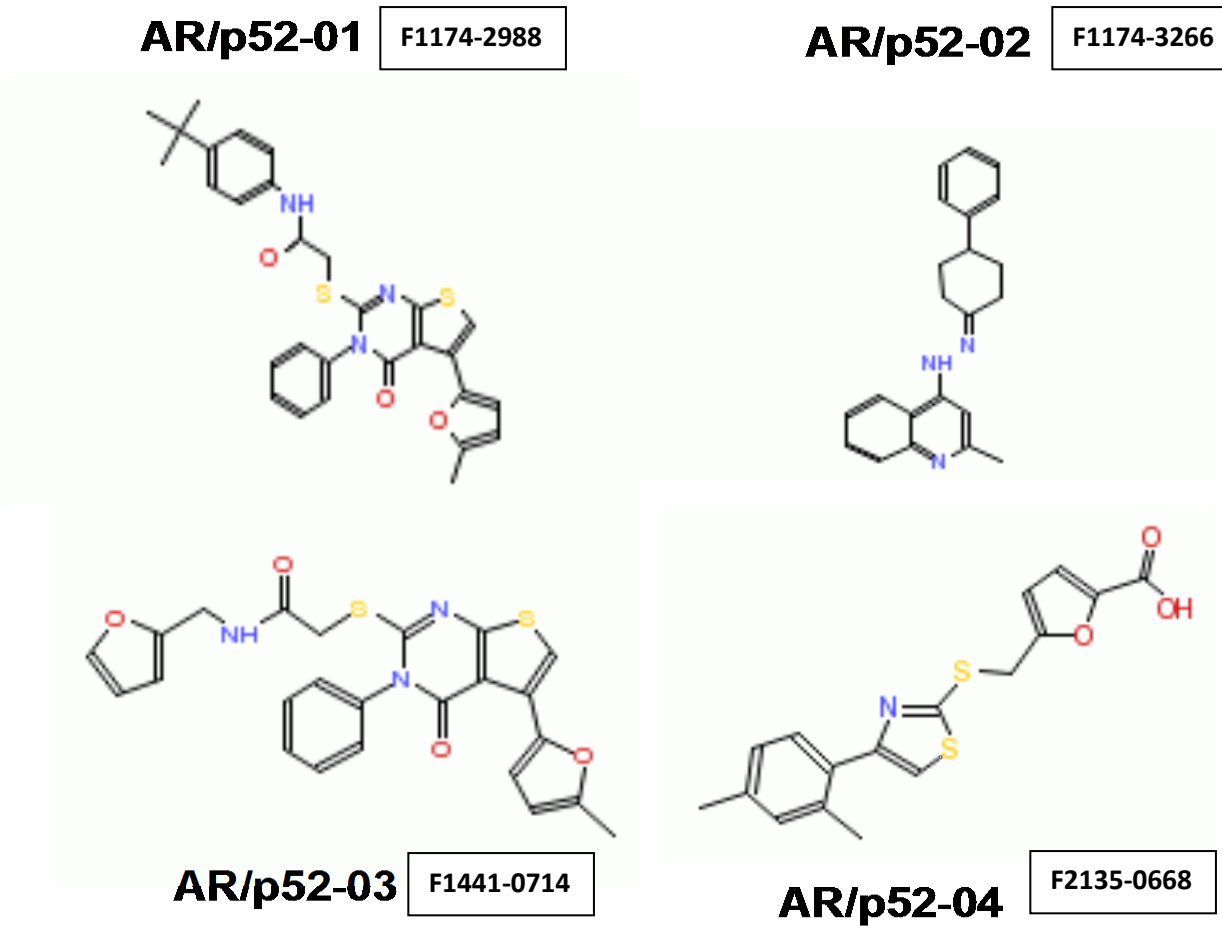

Figure 1. Structure of specific inhibitors of AR and p52 interaction identified by high throughput screen. Structures obtained from Life Chemical Company, compound numbers from Life Chemical shown in boxes.

Table 1. IC50 values for inhibition of castration-resistant LNCaP C4-2 cell growth by AR-p52 inhibitors under androgen deprived conditions. LNCaP C4-2 cells in androgen-deprived medium were treated with varying doses of compound up to 25 μM or zero dose control for 96 h, then harvested for DNA quantitation and analysis of growth inhibition dose response to determine the dose at which growth was inhibited by 50% compared to control (IC50). N=6 data points per dose, experiments repeated. [* Lead compound ARp52-02001 was tested similarly in castration-resistant 22Rν1 cells and found to have an IC50 of 10μM against the 22Rν1 cells (N=6 data points per dose, one experiment).]

Compound	IC_{50} (μM)
ARp52-01001	4
ARp52-02001 *	5
ARp52-03001	10
ARp52-04001	> 25

Table 2. IC50 values for inhibition of androgen-dependent LNCaP cell growth by AR-p52 inhibitors under androgen growth stimulatory conditions. LNCaP cells in medium containing growth stimulatory level of androgen were treated and analyzed as described above to determine IC50s.

Compound	IC_{50} (μM)
ARp52-01001	4
ARp52-02001	5
ARp52-03001	> 25
ARp52-04001	> 25

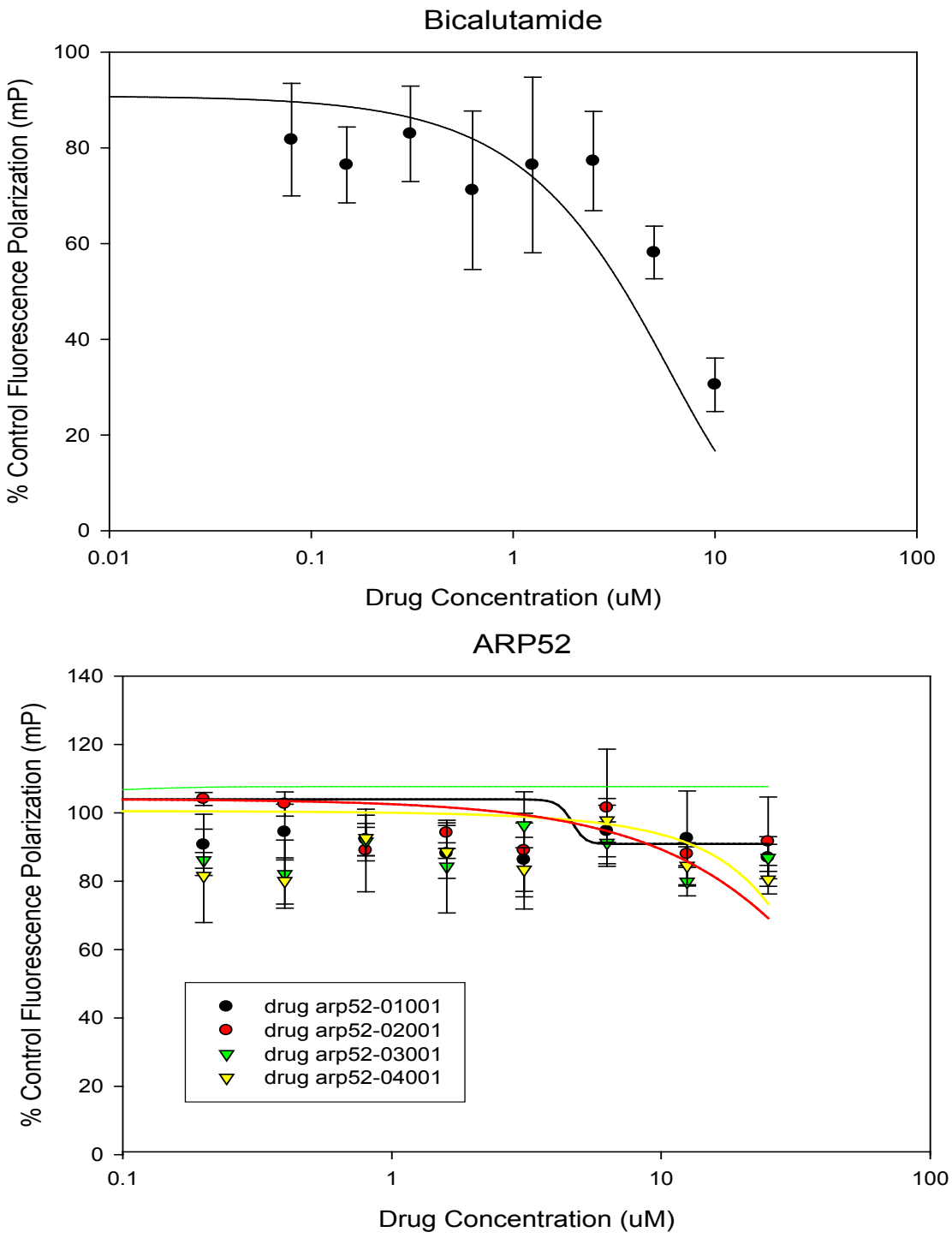

Figure 2. The AR-p52 inhibitors do not bind AR Ligand Binding Domain (LBD). Compounds were assayed using Invitrogen's Polarscreen AR Competitor Assay kit. Clinical antiandrogen bicalutamide showed significant competition with androgen for binding to AR LBD as expected (top graph) while the AR-p52 inhibitors did not (bottom graph).

Table 3. AR-p52 inhibitors at 10μM cause a decrease in LNCaP-C4-2 cells PSA secretion under androgen-deprived conditions. LNCaP C4-2 cells were cultured in androgen-deprived medium and treated with vehicle (control) or 10 μM of compound (N=2 samples per condition). Four days into treatment, media and cells were harvested for determination of PSA secretion per cell. The PSA Enzyme Immunoassay kit from BioCheck, Inc. was used to quantitate PSA in the media, and the ng PSA value was normalized to cell number for each sample. Average ng PSA/cell values for each compound were normalized to the average for Control treated cells (% Control PSA/cell).

Compound	% Control PSA/cell
AR/p52-01	56
AR/p52-02	22
AR/p52-03	60
AR/p52-04	56

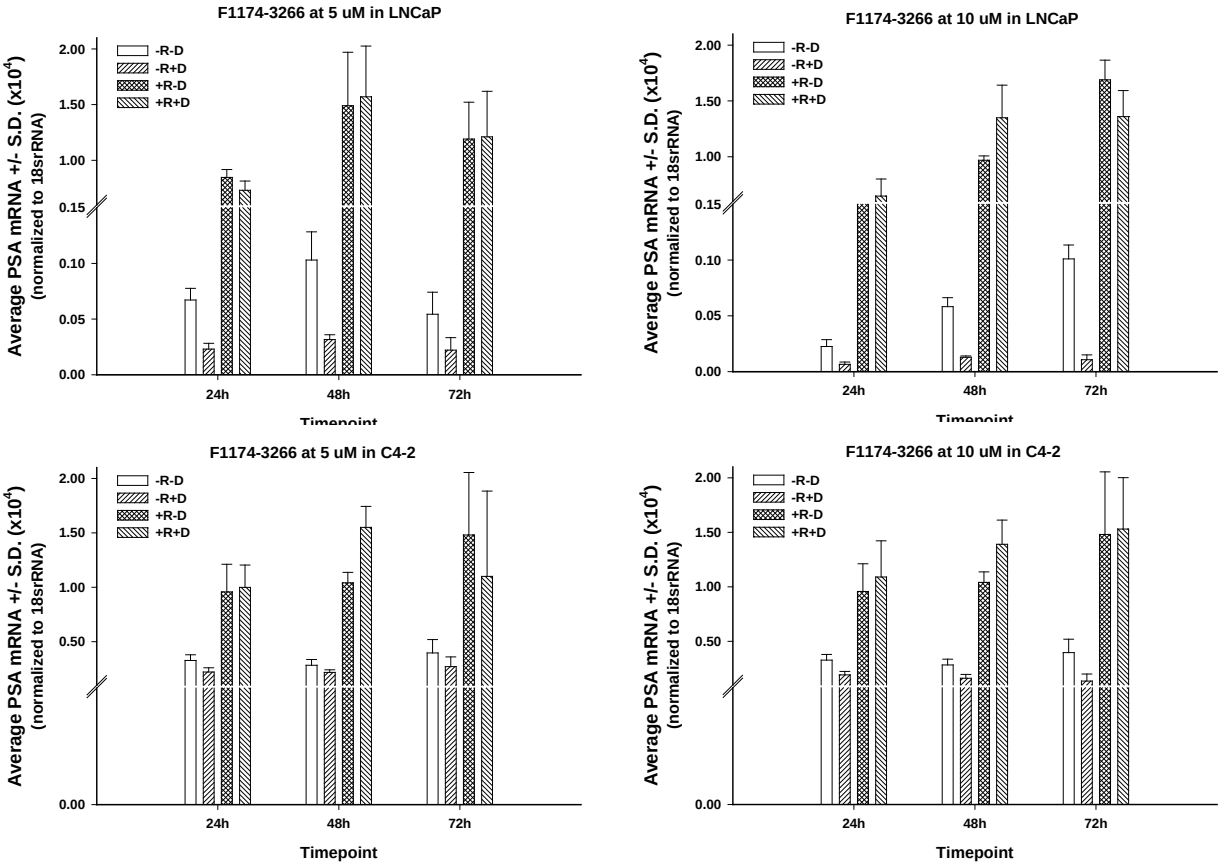

Figure 3. AR/p52-02 decreases PSA mRNA under androgen-deprived conditions in LNCaP and C4-2 cells. LNCaP (top graphs) or C4-2 (bottom graphs) cells were grown overnight in androgen-deprived medium, then treated with 5μM (IC50) or 10 μM of AR/p52-02 (F1174-3266) (D) in the presence or absence (±) of 2nM synthetic androgen R1881 (R). After 72h, the cells were collected using Invitrogen's Cells-to-cDNA kit for quantitative real time PCR analysis using CFX96 instrument (BioRad) to evaluate the level of expression of PSA from each condition. The sequence of PSA primers used are as follows: PSA forward:5'GACCACCTGCTACGCCTCA PSA reverse:5' GGAGGTCCACACTGAAGTTTC. The CT value of PSA was normalized against 18S rRNA cDNA. The sequence of primers used for 18S rRNA in real time PCR were as follows; 18S rRNA forward: 5'CGCCGCTAGAGGTGAAATCT and reverse sequence:5'CGAACCTCCGACTTTCGTT. AR/p52-02 significantly reduces AR transcriptional activity as measured by PSA expression in LNCaP cells under low androgen (F1C4) condition but does not affect androgen induction of AR transcriptional activity: AR/p52-02 significantly reduced PSA mRNA under the androgen-deprived condition (P<0.05 for -R,-D compared to –R,+D at all timepoints) in both LNCaP and C4-2 cells, with greater effect at 10 μM compared to 5μM. As expected, 2nM androgen stimulation led to a significant ~16-fold increase in PSA mRNA (P<0.001 for +R,-D compared to –R,-D at all timepoints) for LNCaP cells. C4-2 cells also responded to androgen stimulation, but more mildly with only ~3-fold increase (P<0.01 for all comparisons). However, AR/p52-02 did not affect PSA mRNA levels under the 2nM androgen-stimulated condition, as no difference was observed at any timepoint for +R,-D compared to +R,+D. Interestingly, baseline PSA mRNA was about 6-fold higher in C4-2 cells compared to LNCaP cells. (N=6 per condition between two experiments.)

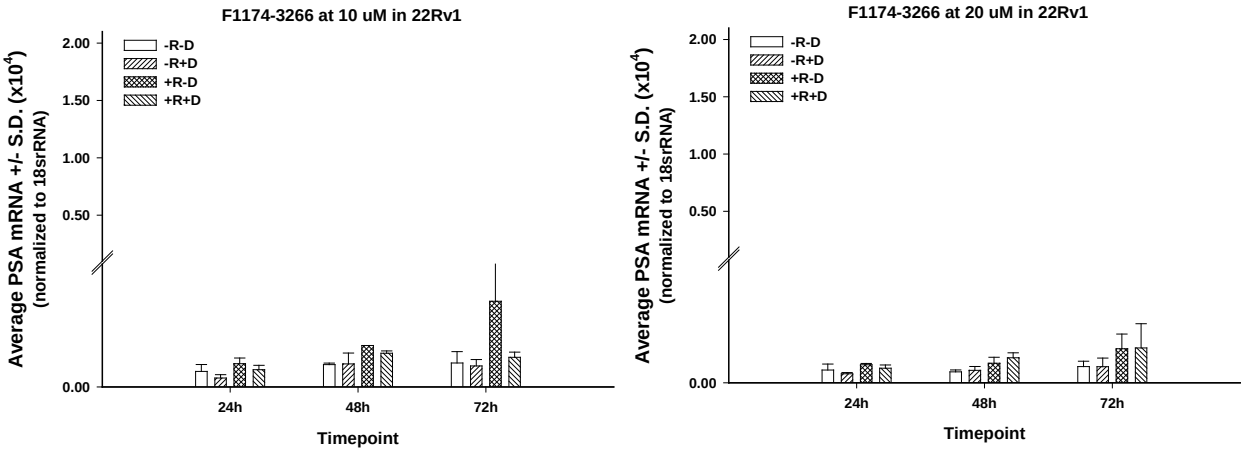

Figure 4. AR/p52-02 does not decrease PSA mRNA in 22Rv1cells. 22Rv1 cells were treated with inhibitor AR/p52-02 (F1174-3266) and analyzed for PSA mRNA as described in Figure 3, N=3 to 6 per condition in separate experiments for treatment with 10 or 20 μM drug (R= androgen R1881, D= drug). Of note, PSA mRNA levels in 22Rv1 cells were significantly lower than in LNCaP cells by 50-fold and C4-2 cells by ~300-fold. Neither drug nor R1881 affected the levels of PSA mRNA in these cells.

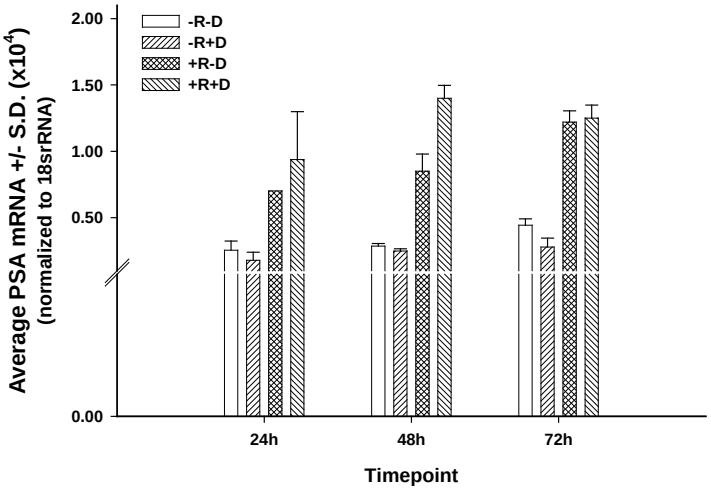

Figure 5. AR/p52-01 does not decrease PSA mRNA in C4-2 cells. C4-2 cells were treated with inhibitor AR/p52-01 (F1174-2988) at 10 μM and analyzed for PSA mRNA as described in Figure 3, N=3 per condition (R= androgen R1881, D= drug). Transcription of PSA was not significantly inhibited by this compound under any condition.

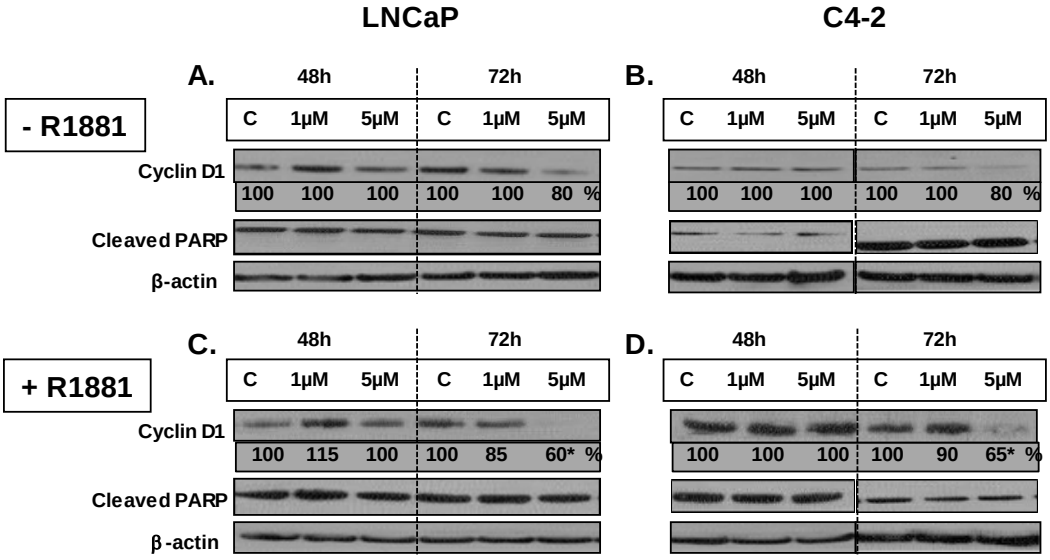

Figure 6. Effect of AR/p52-02 on apoptosis marker (Cleaved PARP) and cell cycle marker (Cyclin D1) in androgen-dependent LNCaP cells and its castrate resistant variant C4-2 cells. LNCaP or C4-2 cells were treated with (+) or without (-) 2nmol/L androgen R1881 and zero (C), 1 or 5μmol/L of lead compound AR/p52-02 (F1174-3266) for 48 and 72h. Cells were then harvested and protein levels were determined by Western blot analysis. β-actin was used as loading control and for normalization. Percentage of each protein band compared to C (**%**) at each timepoint was calculated after normalizing each band against β-actin. Representative blots are shown. The level of Cleaved PARP (89 kda) did not change under treatment conditions. Cyclin D1 (37 kda) level was markedly reduced in R1881 treated LNCaP and C4-2 cells after 72h. The western blots were run three times for cell lysates from three to four different experiments per condition with similar results. Statistical analysis of bands across the experiments showed significantly lower (*****) average cyclinD1 of 65% in LNCaP cells (*P*=0.001, N=3) and 71% in C4-2 cells (*P*=0.05, N=4) compared to control.

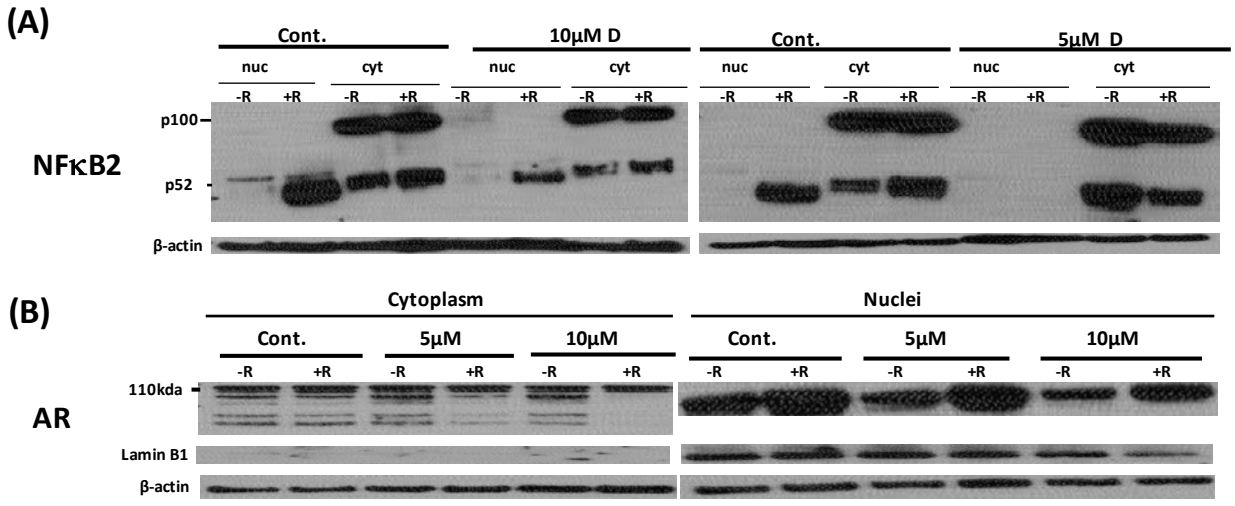

Figure 7 : Nuclei translocation of Androgen Receptor (AR) and NF-κB2 (p52) in LNCaP cells after treatment in the presence or absence of androgen (± 2nM R1881) with 5 or 10uM of AR/p52 inhibitor. (A). In the presence of 2nM R1881, NF-κB2(p52) translocates to the nucleus (compare control lanes in +R with –R), however translocation of NF-κB2(p52) is inhibited by 5μM and 10μM of the inhibitor (D) (compare bands for each treatment with corresponding conrols in each blot). In the cytoplasm, as shown in (**B**), the level of Full length Androgen Receptor (AR) (at 110kda) remains the same for all treatments, however the level of variants of AR have substantially reduced in cells treated with R1881 (+R) and 10μM of the drug. Translocation of AR in cells that were treated with R1881 (+R) and 10μM when compared to control was also reduced. Lamin B1 was used to show the purity of cytoplasmic extraction that was not contaminated with nuclear extracts. β-actin was run as loading control.

Table 5. Levels of AR/p52-02 in mouse plasma over time.

Mice were harvested at designated timepoints following a single dose administration of AR/p52-02 (F1174-3266) in DMSO-saline vehicle (N=2 per condition). Plasma was extracted and LC-MS carried out by a standardized gradient of 2% acetic acid water and 2% acetonitrile.

A. Study 1: 5 mg/kg single dose, Oral or IV

	30 minutes	2 hours	1 week
Oral	**.05 nM**	**.02 nM**	**0**
IV	**3.2 nM**	**1.9 nM**	**0**

B. Study 2: 5 or 50 mg/kg single dose, Oral

	2 hours	24 hours
5 mg/kg	**.1 nM**	**0**
50 mg/kg	**1.0 nM**	**0**

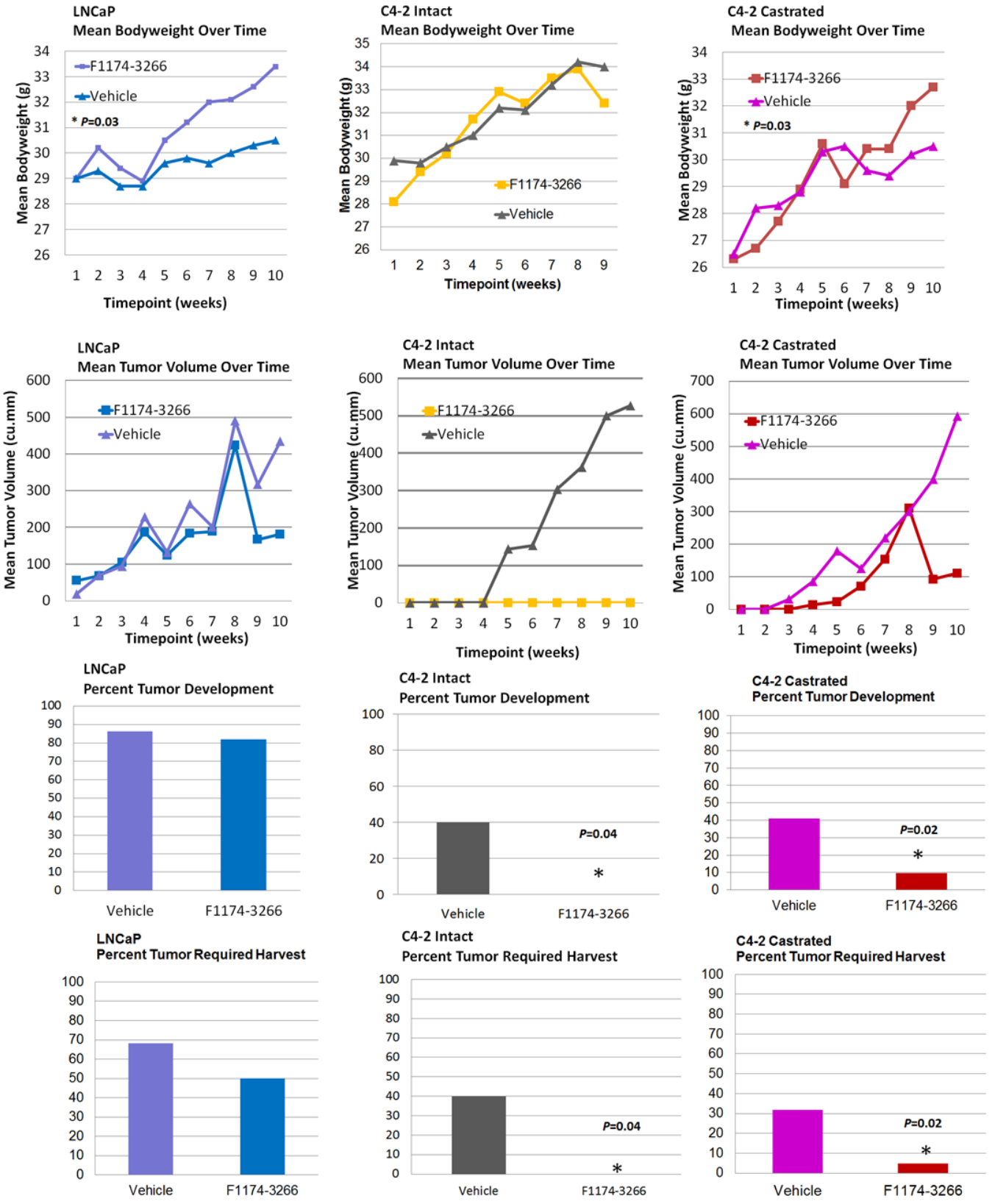

Figure 8. F1174-3266 shows efficacy against C4-2 human prostate cancer xenografts. $1x10^6$ LNCaP or C4-2 human prostate cancer cells were injected in a 50% matrigel DMEM preparation into the left inguinal fat pad of 5-6 week old immuno-compromised aythmic nude mice. For the castrate-

resistant C4-2 model, mice were castrated one day prior to xenograft injection. Starting at two weeks post xenograft, mice were treated orally once daily five days per week with F1174-3266 at the maximum tolerated dose (MTD) of 50 mg/kg or a 60% DMSO saline (vehicle control) solution using a 5 ml/kg body weight dosing volume. Mice were weighed at each treatment and palpated for tumor development and size at least once weekly to measure tumor growth. Efficacy was determined based on body weight and tumor development and growth over time. N ≥ 10 per condition. A repeated measures model was performed on body weight data, and a non-parametric Wilcoxon rank sum test was performed on tumor growth data. A two-tailed heteroscedastic Student T test was used for all other data. Tumor Development: # mice that developed tumors; Tumor Required Harvest: # mice requiring harvest due to tumor size/ decrease in body weight or condition. Bodyweights were significantly higher in LNCaP and C4-2 castrated mice treated with drug compared to vehicle control.Tumor volume over time was not significantly different between vehicle and drug treated groups for LNCaP or C4-2. Percent tumor development was significantly lower in C4-2 intact and castrated mice treated with drug. Percent of mice requiring harvest prior to scheduled end of study due to tumor size and/or bodyweight loss was significantly lower in C4-2 intact and castrated mice treated with drug. Overall, the data shows efficacy of AR/p52-02 against the C4-2 xenograft model, being well tolerated and reducing tumor development and progression.